Transient interaction of cpSRP54 with elongating nascent chains of the chloroplast-encoded D1 protein; 'cpSRP54 caught in the act'

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Abstract **The signal recognition particle (SRP) in bacteria and endoplasmic reticulum is involved in co-translational targeting. Plastids contain cpSRP54 and cpSRP43, unique to plants, but lack a SRP RNA molecule. A role for cpSRP in biogenesis of plastid-encoded membrane proteins has not been firmly established yet. In this study, a transient interaction between cpSRP54 and elongating D1 protein was observed using a homologous chloroplast translation system. Using the novel approach of cross-linking at different time points during elongation of full-length D1 protein, we showed that cpSRP54 interacts strongly with the elongating nascent chain forming two distinct cross-linked products. However, this interaction did not lead to an elongation arrest and cpSRP54 was released from the nascent chains, once they were longer than ~14 kDa. Detailed mutant analysis showed that the cpSRP54 interaction occurred via the first transmembrane domain, which could be replaced by other hydrophobic domains of more than 10 amino acids.**

Key words: cpSRP54; Chloroplast; D1 protein; Translation; Cross-linking

1. Introduction

At least 32 thylakoid membrane proteins are encoded by the plastid genome. A number of these membrane proteins (e.g. D1, D2, photosystem I-A,B) are synthesized at the thylakoid membrane surface on membrane-bound ribosomes [1,2]. The elongating nascent chains are co-translationally inserted into the thylakoid membrane, as demonstrated by accumulation of tightly associated ribosome nascent chain (RNC) complexes and accumulation of translation intermediates in the thylakoid membranes (e.g. [3,4]). A very central question in the expression of the chloroplast-encoded thylakoid membrane proteins, which remains virtually unsolved, is if recruitment of the ribosomal subunits and formation of initiation complexes takes place in the chloroplast stroma or at the (thylakoid) membrane surface. A closely related question is how the nascent chain is directed into the membrane translocon and into the respective complex.

Many components of the targeting and insertion/translocation machinery have been identified in plastids (for recent reviews see [5,6]). Surprisingly, chloroplast signal recognition particle (cpSRP) was shown to lack an RNA molecule found in all SRPs of bacteria, as well as in the endoplasmic reticulum (ER) of eukaryotes. This RNA molecule is central for interaction of SRP with the ribosomes in prokaryotes and the ER (reviewed in [7]). Instead cpSRP54 was found to be associated with cpSRP43 or with the ribosomes. cpSRP43 is unique to plants and the cpSRP54/cpSRP43 complex was shown to be involved in post-translational targeting of a subset of nuclear-encoded LHCP proteins, with the 43 and 54 SRP subunits having an independent activity in vivo but not in vitro [8–11]. Mutants disrupted in both SRP54 and SRP43 have a near total loss of LHCPs and ELIPS [12]. Even more surprising was the finding that SRP and cpFTSY seem to deliver these nuclear-encoded substrates post-translationally to the integral thylakoid membrane protein ALB3, rather than to the cpSecY/E translocon [13,14].

A role of cpSRP54 in (co-translational) targeting of chloroplast-encoded proteins has not yet been established firmly, but ~50% of cpSRP54 can be found associated with chloroplast 70S ribosomes [15]. Interestingly, it seems that cpSRP43 can recruit cpSRP54 from the ribosomes for post-translational targeting activity (Peterson and Henry, unpublished). *Arabidopsis* mutants lacking (functional) cpSRP54 showed a pleiotropic phenotype in plastids of the first young leaves [9,16], suggesting a broad role of cpSRP54 in protein targeting, including targeting of chloroplast-encoded proteins. Stable nascent D1 protein chains, generated in vitro using truncated *psbA* mRNA, could be cross-linked to cpSRP54, as long as the nascent chain remained attached to the ribosome. This interaction depended on the length of the nascent chain that had emerged from the ribosome [17]. Taken together, these data suggest that cpSRP54 is involved in biogenesis of several plastid-encoded thylakoid membrane proteins. However, it can be argued that the formation of stable RNC complexes generated by translation of truncated mRNA could lead to unspecific interactions.

The present study provides further support that cpSRP54 is involved in biogenesis of the D1 protein. During translation of full-length D1, cpSRP54 was found to interact tightly, but only transiently, with the elongating nascent chain, forming two distinct cross-linked products. 'Trapping' this interaction during translation of full-length product, rather than on artificially stable RNC complexes, has not been demonstrated earlier. This approach opens up additional experimental possibilities to probe the role of cpSRP. The requirements for this

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Abbreviations: SRP, signal recognition particle; TM, transmembrane domain; cp, chloroplast; RNC, ribosome nascent chain

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interaction were further investigated by a series of D1-derived mutant constructs.

2. Materials and methods

2.1. DNA constructs

A plasmid containing the full-length 5′ untranslated region (−85 to +1) and the full-length coding region of *psbA* (encoding the pD1 protein) under control of the T7 promoter was used for all wild-type constructs [18]. A number of mutant constructs were used in this study and their construction is summarized in Table 1. All constructs were verified by nucleotide sequencing.

2.2. Preparation of mRNA templates

DNA templates encoding full-length and truncated forms of the D1 protein were prepared by PCR using a forward primer annealing upstream of the T7 promoter and reverse primers annealing in the coding region of the *psbA* gene. In total five different reverse primers were used. Transcription and purification of transcripts was carried out as described in [17].

2.3. Preparation of chloroplast translation extracts, in vitro translations, cross-linking and purification of RNCs

The preparation of a chloroplast translation system from pea leaves was based on [18] as modified in [17]. To prepare translation extract devoid of thylakoid membranes, extracts were prepared by 10 min lysis on ice with repetitive vortexing instead of sonication as described in [19]. Translations were carried out at 30°C as described in [17]. For pulse-chase experiments of full-length D1, translations of full-length *psbA* mRNA were carried out for 8 min at 30°C. Immediately after the pulse, translations were placed on ice and mixed with 50 μM lincomycin and 5 mM cold methionine. Following a 10 min incubation, the 'chase' translation was allowed to proceed at 30°C for up to 30 min. Isolation of RNCs, cross-linking, immunoprecipitations, protein separation and autoradiography were carried out as described in [17].

3. Results and discussion

In an earlier study [17], we investigated the interaction between cpSRP54 and stable RNCs created by translation of truncated *psbA* transcripts of 87, 107 and 189 amino acids without stop codon (Fig. 1A). Chemical cross-linkers were used to cross-link at positions C18 and C125 of the D1 protein and amber suppressor-directed site-specific UV cross-linking at position 48, 53, 60 and 93 [17]. We demonstrated that cpSRP54 interaction with D1 nascent chains is strong when they are 107 aa long but much weaker when they are 187 aa long. This interaction required that the nascent chains were attached to the ribosome.

It can be argued that the accumulation of artificially stable RNCs allows unspecific interactions between SRP and the nascent chains. In this study we tested therefore if this interaction can also be detected during elongation of full-length D1 protein, thus without creating artificially stable RNC complexes. In addition we probed whether (i) TM1 is required for the interaction and if TM1 can be replaced by other less hydrophobic TMs or even short non-membrane-spanning hydrophobic domains and (ii) more efficient interaction with TM2 can be detected either after removing TM1 and/or by introducing additional cross-link sites in TM2.

3.1. Transient interaction between cpSRP54 and the nascent D1 polypeptide

Full-length D1 protein synthesized from exogenous *psbA* transcripts in the homologous pea chloroplast translation system (with the complete 5′ untranslated region) accumulated stable to relatively high levels, as compared to other plastid-encoded gene products such as cytochrome *f* [19] and CFo-III (Rohl and van Wijk, unpublished) (Fig. 1). During elongation, a ladder of D1 intermediates was observed. With increasing chase times, most of these intermediates shifted to higher molecular mass, with full-length D1 proteins accumulating after 5 min, indicating that they represented translation intermediates, rather than truncated products (Fig. 1B). Densitometry plots of the autoradiograms show the relative quantity of each intermediate present at 0, 2.5 and 5.0 min (Fig. 1C). The

Table 1
Constructs used in this study

Name	Description	Stop codon	Length (aa)
pD1	full-length wild-type pD1	yes	352
pD1C18S	full-length pD1, Cys at position 18 replaced by Ser	yes	352
pD1–109	truncated wild-type pD1	no	109
pD1–187	truncated wild-type pD1	no	187
pD1C18S–109	truncated pD1, with Cys at position 18 replaced by Ser	no	109
pD1ΔTM1–91	truncated pD1, TM1 deleted	no	91
pD1ΔTM1–109	truncated pD1, TM1 deleted	no	109
pD1rTM2–111	truncated pD1, TM1 replaced by pD1 TM2	no	111
pD1rTM2–119	truncated pD1, TM1 replaced by pD1 TM2	no	119
pD1rTM5–113	truncated pD1, TM1 replaced by pD1 TM5	no	113
pD1rTMCyt*f*–113	truncated pD1, TM1 replaced by the TM of Cyt *f*	no	113
pD1rHydr–113	truncated pD1, TM1 replaced by a non-TM region of 20 aa	no	113
pD1rHydrM40D/A41R–113	truncated pD1rHydr with two charged residues (Met40 replaced by Asp, Ala41 replaced by Arg)	no	113
pD1V123C–187	truncated pD1, extra Cys in TM2	no	187
pD1G128C–187	truncated pD1, extra Cys in TM2	no	187
pD1ΔTM1V123C–169	truncated pD1, TM1 deleted, extra Cys in TM2	no	169
pD1ΔTM1G128C–169	truncated pD1, TM1 deleted, extra Cys in TM2	no	169

The codon for cysteine residue at position 18 (TGT) was changed to a codon for serine (TCT) resulting in construct pD1C18S. The first TM was removed and an *Nde*I restriction site was introduced using an overlap PCR approach in which amino acids 37–56 were replaced with a histidine residue and a methionine resulting in the construct pD1ΔTM1. TM2 (aa 110–137) and 5 (aa 268–291) and part of the C-terminus (aa 293–312) of pD1, as well as the TM of cytochrome *f* (aa 285–305) were amplified with primers that added *Nde*I sites at both ends. The four fragments were cloned into the pD1ΔTM construct and called pD1rTM2, pD1rTM5, pD1rHydr and pD1rCyt*f*, respectively. To interrupt the hydrophobic domain in pD1rHydr methionine 40 and alanine 41 were replaced with aspartic acid and arginine respectively, resulting in the construct pD1rHydrM40D/A41R. The codons for GTA at position 123 and GGT at position 128 were changed to cysteines in both pD1 and pD1ΔTM resulting in pD1V123C, pD1G128C, pD1ΔTMV123Cand pD1ΔTMG123C.

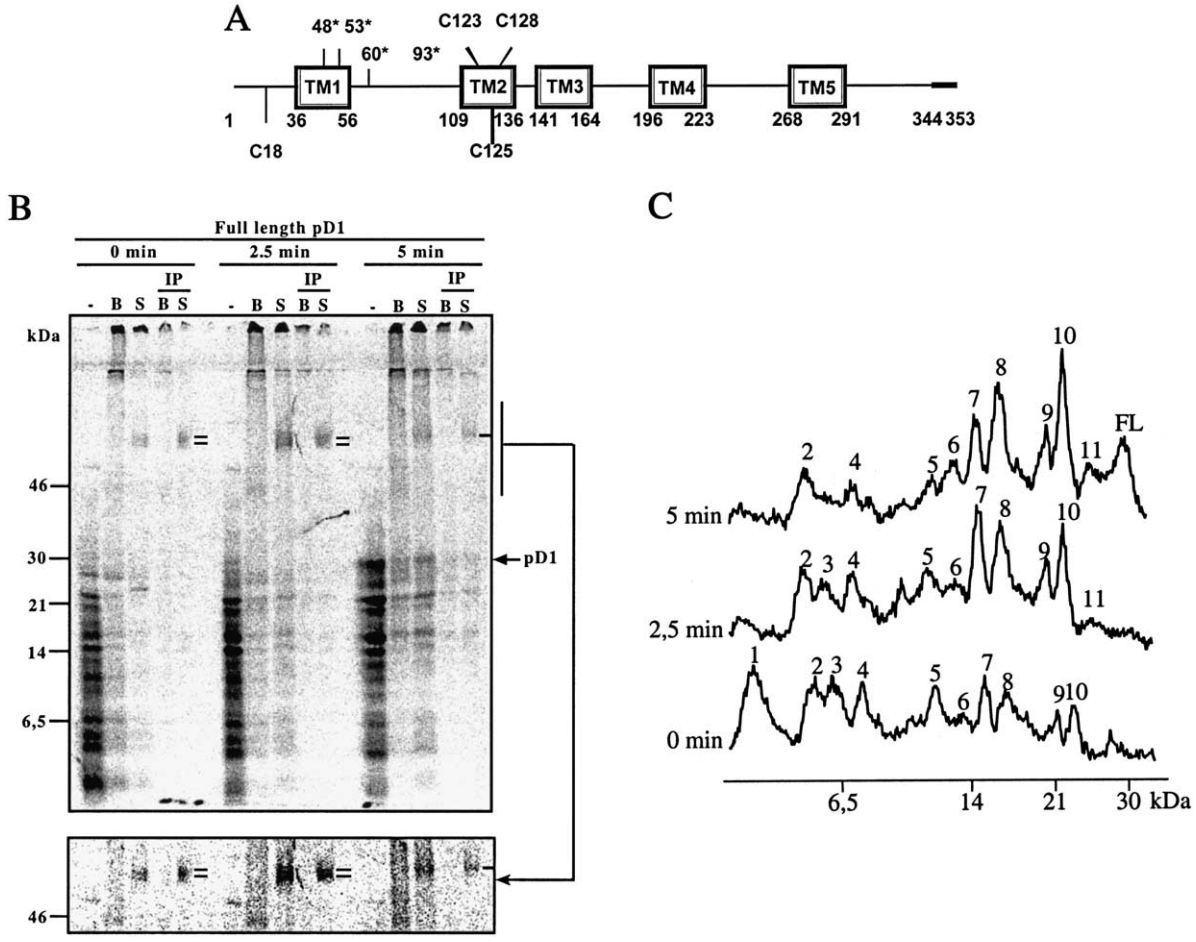

Fig. 1. Transient interaction of cpSRP54 with elongating D1 nascent proteins. *PsbA* mRNAs encoding full-length wild-type pD1 (A) were translated for 8 min, followed by a chase of 2.5 or 5 min as indicated. RNCs were then purified and incubated with BMH or S-MBS. Cross-linked products were immunoprecipitated with cpSRP54 antiserum after denaturing with SDS. Ten percent of the purified RNCs before cross-linking (lanes marked by a hyphen) and after cross-linking with BMH (lanes marked with B) or S-MBS (lanes marked with S) were loaded directly on the gel. The remaining sample was used for immunoprecipitation and precipitated products were loaded (marked with IP). Proteins were separated on Tricine PAGE and analyzed by phosphor imaging. A: Schematic view of the primary sequence of wild-type pD1. Cysteines in D1 are indicated below the line and engineered cross-linking sites are indicated above the line. Transmembrane domains (TM) are indicated and amino acid residue numbering is indicated. Amber suppressor sites used in our previous study [17] are indicated and marked with an asterisk. B: Pulse-chase full-length wild-type pD1. The inset shows a close-up of the cross-linked products at higher contrast. Chase times (0, 2.5, 5 min) are indicated. C: Line plots of the autoradiograms from the experiment presented in B. Translation intermediates are numbered. FL = full-length D1.

transient accumulation of translation intermediates most likely represent pausing intermediates. Ribosomal pausing has been observed in many studies using in organello chloroplast translations and pause sites of elongating D1 protein have been mapped for membrane-bound polysomes in barley chloroplasts by Mullet and coworkers (e.g. [20]). In subsequent papers it was shown that the energy and redox state of the thylakoid membrane exerts a feedback control on elongation ([21,22], reviewed in [23]). However, no experimental evidence has been provided for the actual mechanism of plastid ribosomal pausing. Interestingly, just recently evidence has been presented that a constricted part of the *Escherichia coli* ribosome acts as an exit gate by interaction with a specific sequence motif in the C-terminus of SecM [24]. This interaction between ribosome and nascent chain within the ribosomal structure could regulate pausing or attenuate the rate of further translation. Given the high levels of functional homology between *E. coli* and plastid translational regulation, it is possible that a similar mechanism of translational pausing occurs in chloroplasts.

After purification on sucrose cushions, RNCs and possible associated factors were incubated in buffer (no chloroplast stroma was added) with the cross-linkers BMH and S-MBS, solubilized with SDS and immunoprecipitated with cpSRP54 antiserum. Directly after the pulse, two cross-linked products between cpSRP54 and the radiolabeled D1 nascent chains were detected with the heterobifunctional cross-linker S-MBS and none with the homobifunctional cysteine cross-linker BMH. After 2.5 min of chase, the cross-linked products more than doubled in intensity, whereas their abundance decreased again after 5 min chase time. After 10 min chase no cross-links between D1 nascent chains and cpSRP54 could be detected (not shown). The lower band corresponded to the cross-link product observed when cross-linking with truncated D1–109. The higher band corresponded in size to a cross-link product derived from cpSRP54 and a pausing intermediate of truncated D1–187. The primary sequence of the D1 proteins with transmembrane domains and cross-linking sites is shown in Fig. 1A for convenience. Similar pulse-chase experiments with full-length construct of pD1C18S, in which the

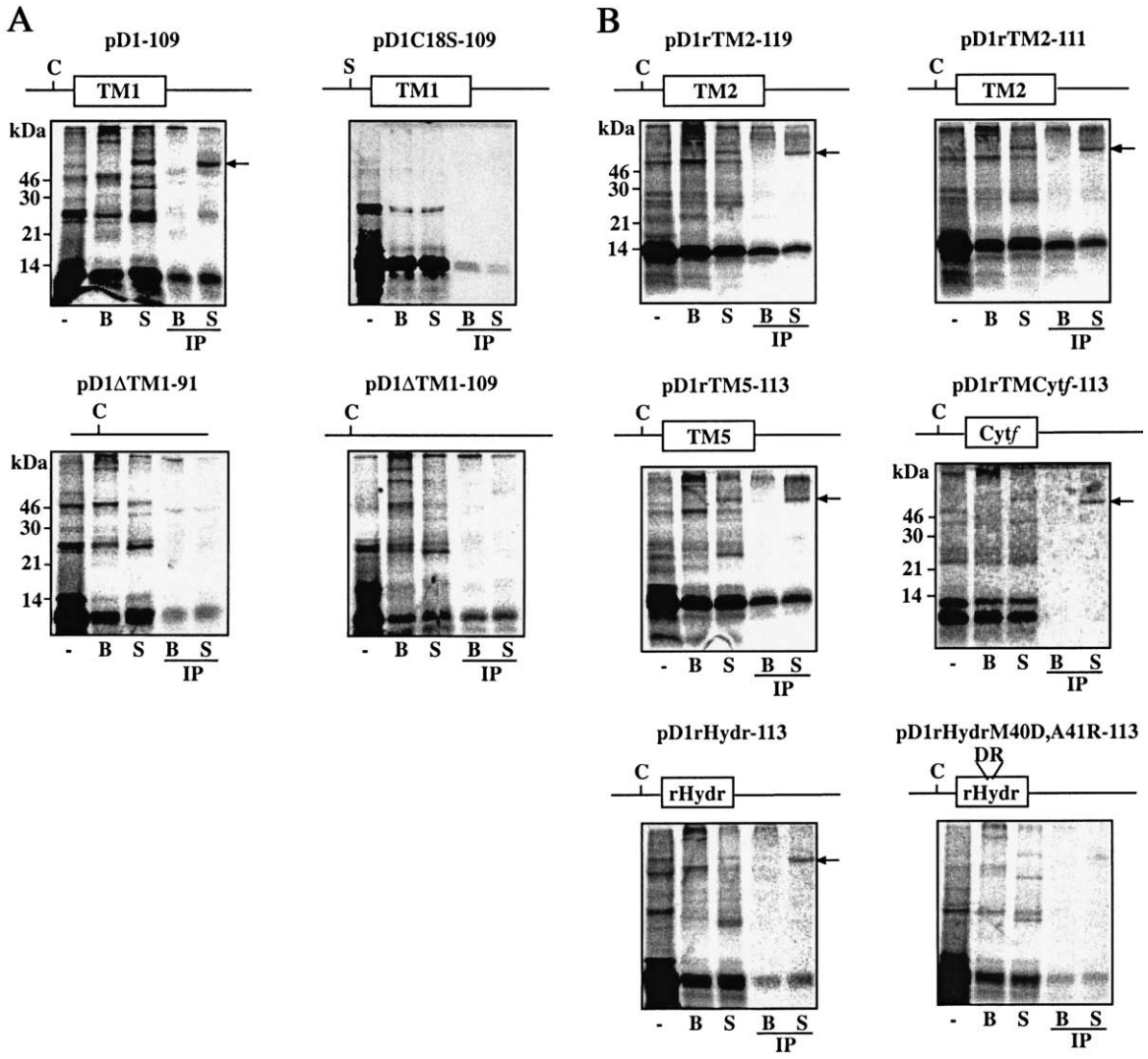

Fig. 2. Determination of sequence requirements for interaction between D1 RNCs and cpSRP54. Truncated *psbA* mRNAs (without stop codon) encoding wild-type and mutant D1 constructs were translated for 30 min and RNCs were subsequently purified on sucrose cushions. RNCs were then cross-linked and immunoprecipitated with cpSRP54 antiserum, as described in Fig. 1. Proteins were separated on 15% Laemmli gels and analyzed by phosphor imaging. A: Synthesis, cross-linking and immunopurification of nascent chains of pD1–109, pD1C18S–109, pD1ΔTM1–91 and pD1ΔTM1–109. B: Synthesis, cross-linking and immunopurification of nascent chains of pD1rTM2–111, pD1rTM2–118, pD1rTM5–113, pD1rTMCyt*f*–113, pD1rHydr113 and pD1rHydrM40D/A41R–113.

cysteine at position 18 was replaced by a serine, did not yield any cross-link product of D1 and cpSRP54 (not shown). In our earlier study, we showed that cpSRP54 serum is highly specific in these immunoprecipitations and precipitations out of chloroplast translation extracts with other sera did not yield any radiolabeled immunoprecipitation products [17].

This transient interaction and formation of distinct cross-linked products shows that the earlier observed interaction of cpSRP54 with D1 nascent chains is not due to the increased time window obtained with artificially truncated *psbA* mRNA [17]. Also, it shows that the interaction is only observed during the initial phase of D1 elongation.

3.2. *CpSRP54 interaction to D1RNC–109 is lost upon removal of the first TM*

cpSRP54 homologues in bacteria and ER of eukaryotes have been shown to interact predominantly with signal sequences or with transmembrane domains located at the N-terminus (reviewed in [7]). This information, together with the cross-link experiments during elongation of full-length D1 (Fig. 1) and combined with our previous study [17], suggests that the first TM (TM1) is required for cpSRP54 interaction. To obtain further evidence that indeed TM1 (aa 37–56) is essential, constructs were made in which the base pairs encoding TM1 were removed by overlap PCR. This introduced an extra histidine and methionine residue and shortened the D1 protein by 18 amino acids. Translation of the wild-type construct of 109 amino acids, followed by purification of RNCs, cross-linking with S-MBS and immunoprecipitation with cpSRP54 antiserum, yielded the typical cross-linked product (Fig. 2A, first panel, last lane), observed before [17]. Replacement of the cysteine at position 18 by serine through site-directed mutagenesis (assigned pD1C18S–109) abolished the cpSRP54 cross-link completely, showing that this cysteine, positioned outside but close to TM1, was the

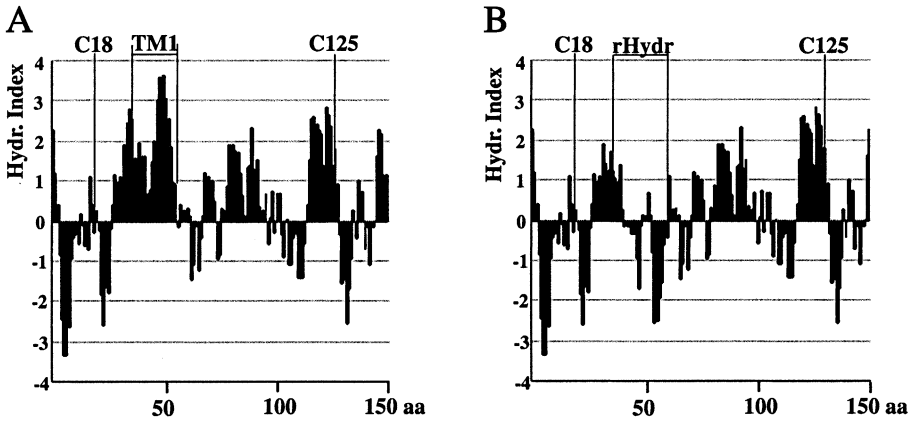

Fig. 3. Hydrophobicity plots. The hydrophobicity of the first 150 amino acids of pD1 (A) and pD1rTM5 pD1rHydr (B) was calculated using the method of Kyte and Doolittle [26] with an averaging window of five amino acids. The cysteine residues at positions 18 and 125 and the first transmembrane domain (TM1) or insert (rHydr) are indicated.

cross-linking site (Fig. 2A). Translation and cross-linking of the truncated construct of 91 amino acid residues, in which TM1 was removed (assigned pD1ΔTM1–91), gave no cross-link with cpSRP54 (Fig. 2A). To ensure that the shorter length of the nascent chain was not responsible for the loss of cpSRP54 interaction, we also created a 109 aa construct without TM1 (assigned pD1ΔTM1–109), using a primer annealing 54 bp further downstream of the encoding region of the *psbA* gene. Also with this construct no cross-link to cpSRP54 could be detected (Fig. 2A). Thus TM1 is indeed the main site of interaction between cpSRP54 and D1 nascent chains.

3.3. CpSRP54 interaction with RNCs only requires hydrophobicity and no specific motif

To study if the very hydrophobic TM1 (of 20 aa) could be replaced by other TMs of lower hydrophobicity, and to exclude the requirement of a specific sequence motif in TM1 important for cpSRP54 interaction (compare the L18 domain in the LHCP family [10]), a number of constructs were created in which TM1 was replaced by other TMs (Fig. 2B). TM1 of D1 was replaced by either the second or the fifth TM of D1 (pD1rTM2 and pD1rTM5) and also by the TM of the plastid-encoded bitopic cytochrome *f* (pD1ΔTMCyt*f*). Truncations of these constructs were chosen to make chains of comparable

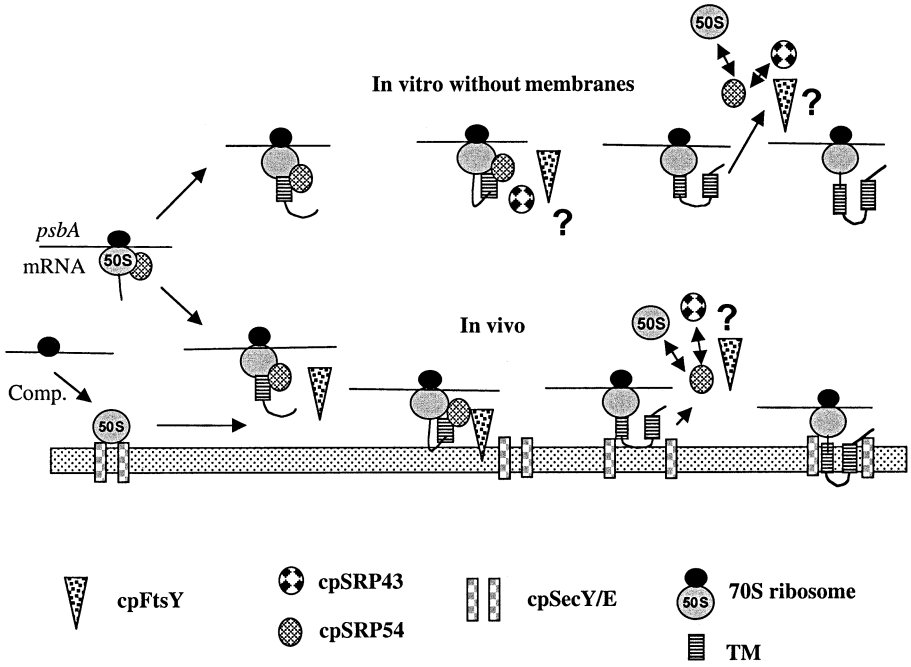

Fig. 4. Schematic outline of cpSRP54 involvement in D1 biogenesis. After translation initiation and during emergence of the first TM of the D1 protein out of the ribosome, cpSRP54 (already interacting with the ribosome) tightly binds to TM1. Interaction of cpSRP54 with cpFtsY directs the nascent chain complex to the SecY/E translocon. cpSRP54 and cpFtsY release upon GTP hydrolysis and cpSRP54 possibly associates with cpSRP43 or with 'empty' ribosomes. In the absence of membranes, cpSRP54 loses its affinity for the D1 nascent chains upon continued elongation and either remains associated with the ribosome or releases and binds to cpSRP43. D1 RNC complexes were found to interact with cpSecY [27] and cpSecY,E and Alb3 all co-purify with thylakoid membrane extracted RNCs (Marbach, Nilsson and van Wijk, unpublished). The natural affinity of the large subunit of the ribosomes for the membrane and its protein components, such as the SecY/E translocon, as observed for bacteria and ER [25], might provide a compensation mechanism in case of absence of cpSRP54 (comp.).

length (between 111 and 119 aa), as summarized in Table 1. Since TM2 (of 28 aa) is significantly longer than the other three TMs, we created nascent chains of two different lengths for this construct (pD1rTM2–111, pD1rTM2–119), to ensure that the cross-linking results were not affected by the length of the nascent chain. Transcription and translation of these four constructs yielded stable RNCs and cross-linking and immunoprecipitation with cpSRP54 gave a clear cross-linked product of the nascent chain with cpSRP54 for all constructs of comparable intensity (Fig. 2B). This indicated that different TMs can replace D1-TM1, and that D1-TM1 is unlikely to contain any specific sequence information required for cpSRP54 interaction.

We then also replaced TM1 by a 20 amino acid domain from the soluble C-terminus of D1 (assigned pD1rHydr). The hydrophobicity plot indicates that even though this removed the hydrophobic TM, there was still a short hydrophobic 'patch' of about 14 amino acids between residues 29 and 43, generated by hydrophobic residues in the wild-type N-terminus and some residues of the insert (Fig. 3). Transcription and translation of this truncated construct of 113 aa still gave a weak cross-link to cpSRP54 (Fig. 2B), indicating that the remaining hydrophobic domain was still sufficient for weak interaction with cpSRP54. To further decrease the hydrophobicity of this region, the methionine and alanine residues at positions 40 and 41 were replaced by two charged residues (aspartic acid and arginine), thereby reducing the hydrophobic domain to less than 10 residues (assigned pD1rHydrM40D/A41R) (Figs. 2B and 3). This resulted in an almost complete loss of affinity for cpSRP54.

3.4. Can cpSRP54 also interact with TM2?

Our earlier study using both amber suppression-directed UV cross-linking and chemical cross-linking clearly showed that the interaction between TM1 and cpSRP54 was lost as elongation proceeded to position 189. There is the possibility that an interaction between TM2 and cpSRP54 took place after the release of cpSRP54 from TM1. However, no significant cross-linking between C125 in TM2 and cpSRP54 could be obtained with translated D1–189. To probe this in more detail, we engineered additional cysteines into TM2 at positions 123 and 128, both in wild-type D1 constructs (pD1V123C–187, pD1G128C–187), and in constructs in which TM1 was removed (pD1ΔTM1V123C–169 and pD1ΔTM1G128C–169) (see Table 1). Autoradiograms of translation and cross-linking experiments did not show any significant induction of new cross-linked products in the four constructs, indicating that indeed TM2 does not engage in interaction with cpSRP54 (data not shown).

4. Conclusions

In conclusion, cpSRP54 and D1 interact during the early phase of D1 elongation. This interaction (i) requires that the nascent chain is still attached to the ribosome, (ii) requires a hydrophobic domain of more than 10 amino acids, but does not specifically require TM1, (iii) involves TM1 and is gradually lost as elongation proceeds to the point where it is not detectable when the nascent chain is 187 amino acids, (iv) does not take place with TM2 and thus the interaction with cpSRP54 is not transferred from TM1 to TM2, (v) with cpSRP54 is lost during progressive elongation in the absence of thylakoid membranes, possibly via interaction with cpSRP43 or with soluble cpFtsY, and (vi) does not lead to any elongation arrest by cpSRP54, which is consistent with the absence of a cpSRP RNA moiety and the absence of additional subunits (homologues of SRP9 and SRP14) that function in elongation arrest of ER-targeted proteins. These conclusions are summarized in a working model depicted in Fig. 4.

These results, together with earlier observations (reviewed in [23]), suggest that cpSRP54 assists in early steps of D1 biogenesis and plays a role in directing the first transmembrane domain of the D1 protein to the SRP receptor cpFtsY and the cpSecY/E (and possible Alb3) translocon (Fig. 4). There is a fairly narrow window within which SRP54 can operate since SRP54 loses its affinity as elongation progresses. As depicted in Fig. 4, it is possible that cpSRP43 competes with the RNC complex and 'empty' ribosomes for interaction with cpSRP54, to engage in post-translational targeting of the LHCP and Elip families. This interaction might also provide stability of cpSRP54 when it is not associated with ribosomes. The natural affinity of the large subunit of the ribosomes for the membrane and its protein components, such as the SecY/E translocon, as observed for bacteria and ER [25], might provide the compensation mechanism for a loss of cpSRP54 in *Arabidopsis* mutants lacking cpSRP54.

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